Progress Report -FY 2006

Core Name: Pathogen Source Tracking

Project Title: Pathscan and Pathcast: Towards predicting the distribution and abundance of zoonotic pathogens in the marine/estuarine environment.

Reporting Period: 1 October, 2005 – 30 September, 2006

Principal Investigator(s): John Jacobs, CCEHBR/Oxford, Jan Gooch, CCEHBR/Charleston, Andy Depaola, USFDA/ GCSL, George Blackstone, USFDA/ GCSL, Gretchen Messick, CCEHBR/Oxford, Bob Wood – CCEHBR/Oxford

Associate Investigator(s):

Background and Rationale:

There is increasing evidence that anthropogenic influences and environmental degradation impact the distribution, abundance, and virulence of waterborne pathogens (Henrickson et al. 2001). Human enteric pathogens are known to cause widespread waterborne disease in the United States both through ingestion of contaminated seafood and direct water contact, costing an estimated $20 billion a year in lost productivity (CDC, www.cdc.gov). For example, in 1999 alone 342 cases of confirmed non-cholera *Vibrio* infections were reported to the Center for Disease Control through their *Vibrio* Surveillance System. Fourty-six percent of these cases were hospitalized and 11% resulted in patient death. While *Vibrio parahaemolyticus* was most prevalent, 94% of the mortalities were associated with *V. vulnificus.* While monitoring human health and disease outcomes is important, protecting human health and the economic value of coastal and estuarine environments and resources requires the development of rapid pathogen detection and forecasting tools.

Traditional identification techniques for pathogenic bacteria are labor intensive and time consuming because long incubation periods and selective media are required to culture these organisms (Lyon, 2001). With the advent of the Polymerase Chain Reaction (PCR), however, the ability to screen for pathogens in environmental samples has been greatly enhanced. Unfortunately, simply detecting these pathogens in the environment is often of limited value, as many are a normal component of healthy ecosystems. The need to rapidly quantify pathogens, and virulent strains, has begun to be addressed by the development of real-time or quantitative PCR which utilizes a fluorescent reporter to quantify the exponential increase in PCR product with each cycle. In addition, coupling real-time PCR with multiplex PCR, or the use of several primers and fluorescent reporters simultaneously, offers the ability to screen and quantify multiple species or multiple genes within a species rapidly (in hours), with out the use of culture methods (days) (Blackstone et. al, 2003). This is particularly beneficial in the quantification of slow growing organisms such as many *Mycobacterium* spp. (weeks to months) and viable but non-culturable states.

While molecular techniques offer a mechanism for rapid screening and reporting of pathogens in water, a further understanding of conditions which favor growth of specific pathogen species and virulent strains is of great importance in the development of predictive capabilities. Thus, this project will examine linkages between water quality, land use, climatology, and zoonotic pathogen abundance and distribution in the environment and host organisms. We envision this project as a catalyst for a larger scale Coastal Pathogen Monitoring Program that would be supported by strategic partnerships among state, federal, academic, and NOAA research and management programs. This work will be conducted through a partnership with the USFDA Gulf Coast Seafood Laboratory (GCSL), the Cooperative Oxford Lab and its partners (NOAA/NOS/CCEHBR, NOAA/NMFS/NCBO, and the Maryland Dept. of Natural Resources), the USDI National Park Service, and the South Carolina Department of Natural Resources.

Objectives:

- Develop and evaluate user friendly, real-time PCR techniques for the quantification of species of *Vibrio* and *Mycobacterium* from water samples and blue crab hemolymph;
- Provide real-time abundance information for select pathogens to State Health agencies (PATHSCAN);
- Develop and evaluate forecast models that predict pathogen abundance and distribution relationships using water quality, land use, and climatological data (PATHCAST);
- Transfer technology to Coastal states and Health agencies for implementation where applicable.
- Develop outreach and education products that depict model results in the form of conceptual models and newsletters written for students, educators, and the interested public.

Accomplishments:

Methods development:

- **Developed and evaluated user friendly protocols for concentrating and extracting of DNA from estuarine water samples.** For the purpose of quantifying DNA from environmental samples, the process of removing and concentrating DNA must be efficient, highly repeatable, and remove substances that interfere with the chemistry of the reaction. Our protocol was developed by modifying a commercially available kit to increase cell lysis and removal of bacteria from filters, and reduce PCR inhibitors. Results indicate 50% greater yield than other protocols examined ($p = 0.0001$), high repeatability from replicated environmental samples (paired t-test, $p = 0.766$), and complete removal of PCR inhibitors.

- **Optimized assays for quantifying both virulent and environmental strains of *Vibrio parahamolyticus (Vp), Vibrio vulnificus (Vv)* and also for the genus *Mycobacterium.*** The approach used in this project represent a methodological improvement on the standard polymerase chain reaction (PCR). As with conventional PCR, the assays target specific gene segments and amplify the region through repetitive cycles of denaturing double stranded DNA, annealing of primers, and extension until sufficient copies exist to detect its presence. Real-time PCR differs from conventional in that the accumulation of product is monitored in "real-time", rather than examined on a gel once the reaction has terminated. In our case, this is accomplished with the use of fluorescently labeled probes. The probe binds to the target region, and is released into suspension each time product is produced. The number of product generating cycles it takes to detect an exponential increase in florescence in the sample is directly proportional to how much target DNA was initially in the sample. Thus by developing standard curves with known quantities of target, the assays can be used to quantify genes in environmental samples.

    - *Vp* – The *Vp* assay was developed by our partners at the GCSL. The assay simultaneously targets a gene specific for the species, and two others which are associated with virulence (thermostable direct hemolysin (tdh) and thermostable related hemolysin (trh). In addition, the assay incorporates a unique internal control which allows for the detection of inhibitors and false negatives. By optimizing and using this assay for total *Vp* and virulence markers separately, we can quantify from 10^6 to 0.5 cells/ml in a 200ml water sample. In addition, this approach saves reagents, as only those positive for *Vp* are further evaluated for virulent strains.
    - *Vv* – Primers and probe for the *Vv* assay targeting the species-specific cytolysin-hemolysin gene (Vvh) were obtained from the literature, evaluated for specificity, and reaction conditions optimized. As above, the assay incorporates and internal control and is capable of quantifying 10^6 – 1 cell/ml in a 200 ml water sample.
    - *Mycobacterium* – Primers and probe for the *Mycobacterium* genus level assay were obtained from the literature targeting a segment of an internal transcribed spacer region (ITS). The assay has been extensively evaluated for specificity and optimized allowing for the quantification of 10^6 – 10 cell/ml.

- **Initiated adaptation of assays for the quantification of *Mycobacterium avium,* and separation of fast and slow growing mycobacteria.** The ITS region targeted for our genus level assay is variable among species. Currently, we have obtained many isolates of mycobacterial species representing both fast and slow growing species and have demonstrated that the assay is capable of distinguishing between these groups based on product size. The variability of the ITS may allow for further development of species specific probes. *Mycobacterium avium* causes disseminated infection in humans and many animals, particularly those who are

immunocomprimised. A probe obtained from the literature designed to work with the ITS target region is under evaluation.

- **Developed culture and probe based assay for the presence of *Vp, Vv* and virulent strains in blue crab hemolymph. -** Several vibrio species are commonly isolated from the blue crab (*Callinectes sapidus*), one of the most important recreational and commercial species in the mid-Atlantic region. By coupling with an existing survey of parasites in this crustacean in Maryland's coastal bays, we hope to examine how vibrio loading, and specifically virulent strains, in the organism relates to water concentration, water quality, and other environmental parameters. Initial efforts have concentrated on direct plating of known hemolymph volumes on selective vibrio agar. Colonies are lifted and identified as *Vp* (virulent vs. non-virulent), *Vv*, or other. Current efforts are targeting direct molecular quantification from hemolymph.

Environmental Monitoring:

- **Completed 12 month pathogen survey (August 05 - August 06) for *Vp, Vv* and *Mycobacterium spp*. in Maryland and Virginia's Atlantic Coastal Bays in collaboration with the National Park Service's Water Quality Monitoring Program.** The National Park Service monitors 18 stations monthly for 16 physical and chemical water quality parameters. By joining this survey, surface water samples are collected for pathogen abundance at the same time as those for water quality. Preliminary analysis has been completed for a portion of the data and results are listed below in Modeling/data analysis.

- **Expanded spatial resolution of above survey from 18 to 44 sites monthly(August 2006) as prescribed by success of initial land use-water quality-pathogen abundance modeling and statistical analysis (see *Modeling/data analysis* section below).** The Maryland Department of Natural Resources maintains an additional 26 stations for monthly water quality monitoring. Samples have been collected in conjunction with this program since August, which has expanded the spatial distribution and intensity of our monitoring. In addition, the inclusion of these stations intensifies the development gradient we are examining in modeling efforts.

- **Completed sampling of Chesapeake Bay Maryland waters in conjunction with the Chesapeake Bay Program's water quality monitoring network (83 sites sampled over a 1 week period).** In August of 2006, all of Maryland's tidal fixed Chesapeake Bay water quality monitoring stations were sampled in a similar fashion as above. This effort was initially conducted to provide a quantitative baseline abundance "Bay snapshot" of key pathogens during peak water temperatures, and to demonstrate the feasibility of our approach over a large spatial scale. Samples will be collected quarterly beginning in January of 2007.

- **Completed sewage treatment site monitoring in coordination with OHHI project "Molecular Techniques for Pathogen Detection"** – The OHHI project "Molecular Techniques for Pathogen Detection" of Lewis et al. conducted sampling for *Cryptosporidium* and fecal coliform bacteria in water surrounding three sewage treatment facilities from May to July of 2006. Split samples were taken for quantification of *Mycobacterium* because of their elevated resistance to chlorine disinfectants and to examine relationships with fecal indicator bacteria. Results indicate a high degree of variability among locations, however, data analysis is incomplete at this time.

- **Completed survey of *Vibrio* spp. in hemolymph of approximately 400 blue crabs collected from Maryland and Virginia coastal bays (Sept. 05-Aug 06).** In collaboration with an existing blue crab monitoring program in Maryland's coastal bays, we monitored blue crab hemolymph from September to August for Vp and Vv using the assay described above. Data collected from this project will be examined for the relationship of water concentrations to those of the crab hemolymph as well as land use, water quality, and other environmental parameters.

Modeling/data analysis

- **Identified and quantified *water quality/pathogen* and more detailed *land use/water quality/pathogen* relationships using multivariate statistical models with sub-watershed resolution of the Maryland/Virginia coastal bay ecosystem.** Preliminary analysis of Coastal Bays data has proven the utility of this approach. We have applied linear regression and spatial modeling to pathogen abundance, water quality, and land use data segregated by sub-watershed (12 digit HOC). Preliminary results suggests that over 40% of the variability in *Vp* abundance can be explained by the linear combination of temperature, pH, and turbidity, with elevated densities positively correlated with developed lands. *Mycobacterium spp.* shows a strong eutrophication signal. Principal component analysis suggests a strong association with environments containing high chlorophyll and nitrogen compounds, having higher water temperature, and low dissolved oxygen and station depth. *Mycobacterium spp.* were not associated with environments having elevated wetland coverage or barren land (beaches) (Figure 1). Full analysis of Coastal Bays data including *Vibrio vulnificus* will be conducted this Fall in preparation for publication.

Other

- **Co-Hosted USGS/NOAA community workshop on mycobacteriosis in striped bass that brought together regional scientists and managers to characterize the current state of knowledge and prioritize future research direction.** In May of 2006, USGS and NOAA convened 40 regional scientists and managers to evaluate the state of knowledge regarding mycobacteriosis in Chesapeake Bay. Participants included representatives from several line offices of NOAA, USGS,

and USFWS, regional scientists actively working on the issue, and State management representatives. The three day workshop was refereed by an expert review panel and resulted in the direct transfer of the most current information to management agencies and a detailed work plan for collaborative efforts over the next 5 years. Proceedings are in press, and available electronically at http://www.nccos.noaa.gov/.

Publications/Presentations:

Jacobs, JM 2005. qPCR: Principles and Application to Environmental Samples. Interstate Seafood Seminar, Ocean City, MD, October 10-12.

Jacobs, J., Messick, G., Sturgis, B., Blackstone, G., Rhodes, M., Gooch, J., and Depaola, A. 2006. Development and application of Real Time PCR for detection and quantification of *Vibrio parahaemolyticus* in coastal waters. NOAA/OHHI Principal Investigators Meeting, Charleston, SC, January 18-20. (Poster)

Jacobs, J., Depaola, A, Blackstone, G., Gooch, J., Messick, G., and Wood, B. 2006. Pathscan and Pathcast: Towards predicting the distribution and abundance of zoonotic pathogens in the marine/estuarine environment. Project summary book, NOAA/OHHI Principal Investigators Meeting, Charleston, SC, January 18-20, pp. 76-77.

Jacobs, J., M. Rhodes, B. Sturgis, B. Wood, and K. Greenhawk. 2006. Bacterial pathogen abundance in relation to land use and water quality in the Coastal Bays watershed, Maryland and Virginia, USA. The Society of Wetland Scientists 27th International Conference and the Australian Marine Sciences Association 44th Annual Meeting, 9 - 14 July, 2006, Cairns, Queensland, Australia. (Oral Presentation and published abstract, p. 60).

Jacobs, J., and Ottinger, C. 2006. Mycobacteriosis in wild marine fishes: A synthesis of current understanding. The Australian Marine Sciences Association 44th Annual Meeting, 9 - 14 July, 2006, Cairns, Queensland, Australia. (Oral Presentation and published abstract, p. 59).

Messick, G., Jacobs, J., and Filipowicz, J. 2006. An overview of disease surveys in crustaceans from the Chesapeake Bay and United States mid-Atlantic coast. 31st Annual Eastern Fish Health Workshop, 27 – 31 March, Mt. Pleasant, SC. (Oral Presentation and published abstract).

*Ottinger, C. and Jacobs, J. 2006. USGS/NOAA Mycobacteriosis in Striped Bass Workshop. USGS Scientific Investigations Report Series #206-5214: NOAA NOS Technical Memo Series #41

* Author or co-author of 7 contributed papers to this workshop and proceedings

Application/Technology Transfer relevant to OHH Strategic Goals

1.0 Scientific Research and Application

This research is centered on the development and application of new molecular tools to assess, report, and potentially predict the distribution and abundance of select pathogens in estuarine and marine waters. Combining near real time pathogen monitoring with existing water quality programs provides a value added means for obtaining detailed physical and chemical profiles in combination with pathogen abundance. Modeling exercises to be performed in later years of this project will incorporate this site specific information as well as land use and climatic data.

2.0 Public Information and Outreach

A critical component of this research is developing the capability to provide near real time information on select pathogen abundance to agencies charged with protecting human health. We have established relationships with State health officials in Maryland and Virginia in order to electronically submit quantitative data for their use in public health management. While our sampling efforts will incorporate good spatial and temporal coverage, much more can be gained by establishing a network of laboratories using standardized methods. We envision this research as a catalyst for such a network, and thus plan to hold workshops and training events for Federal, State, and Academic personnel once methods have been fully evaluated.

3.0 Capacity Building

Through this initiative, the NOAA/NOS Oxford Laboratory has greatly enhanced its molecular and microbiology capabilities. Internal support of OHHI goals has lead to the establishment of new facilities to safely conduct research and monitoring efforts with class II pathogens; a capacity that did not previously exist. In addition, training of principle investigators and technical staff has transformed our capabilities in this area from outsourcing a large proportion of this type of work, to the demonstrated ability to conduct large-scale monitoring efforts.

Project abstract:

Bacteria of the genus *Vibrio* and *Mycobacterium* can cause illness ranging from gastroenteritis and localized wound infections to septicemia and mortality through either ingestion or handling of contaminated seafood, or direct water contact. While the ability

to quickly quantify distribution of human pathogenic bacteria in aquatic sources is of great importance in protecting user groups, traditional culture based techniques are often extremely labor intensive and can require extensive incubation periods negating rapid reporting and reducing sample size. To address this issue, we are working with the USFDA Gulf Coast Seafood Laboratory, and the Center of Excellence for Oceans and Human Health at the HML Pathogen Source Tracking Core to develop and evaluate user friendly, real-time PCR techniques for the quantification of species of *Vibrio* and *Mycobacterium* from water samples and blue crab hemolymph. Real-time PCR uses a fluorescent reporter to quantify the exponential increase in PCR product with each cycle while offering the ability to screen and quantify multiple species or multiple genes within a species rapidly (in hours), with out the use of culture (days –weeks). These assays will be applied in conjunction with State and Federal water quality monitoring programs in the Chesapeake Bay, Maryland and Virginia Coastal Bays, and waters of South Carolina to provide real-time abundance information for management agencies to reduce the risk of human infection from waterborne or seafood related pathogens. In addition, this approach will allow for the examination of relationships of pathogen abundance and distribution with water quality, land use, and climatology for the potential development of predictive models.

To date, three assays have been optimized and evaluated for sensitivity for *Vibrio parahaemolyticus* (including total, and 2 virulence markers), *Vibrio vulnificus*, and *Mycobacterium* spp. All assays currently incorporate a unique internal control developed by our USFDA partners which prevents false negatives and allows for adjustment of quantitative data in the presence of minor PCR inhibition. In addition, concentration and extraction protocols have been developed allowing for direct quantification of these pathogens from 200 ml of filtered estuarine water without inhibition. Highly linear, and repeatable standard curves have been produced for each assay from extraction using this protocol. The assays have been applied through monthly sampling of Maryland's Coastal Bays from August 2005 through present in conjunction with the National Park Service water quality monitoring program (18 stations per month). From August 2006 to present, an additional 26 stations have been added to increase spatial resolution in the Coastal Bays. Again in August, samples were taken (n=83) in conjunction with the Maryland Department of Natural Resources water monitoring program representing the entire Maryland portion of the Chesapeake Bay. This sampling will proceed quarterly in FY07. In addition, methods have been developed and applied to determine the prevalence of *Vibrio parahaemolyticus* in blue crab hemolymph collected in conjunction with our Coastal Bays monitoring. Future work will attempt to apply all assays directly to crab hemolymph.

Preliminary analysis of Coastal Bays data has proven the utility of this approach. We have applied linear regression and spatial modeling to pathogen abundance, water quality, and land use data segregated by sub-watershed (12 digit HOC). Preliminary results suggests that over 40% of the variability in *Vp* abundance can be explained by the linear combination of temperature, pH, and turbidity, with elevated densities positively correlated with developed lands. *Mycobacterium spp.* shows a strong eutrophication signal. Principal component analysis suggests a strong association with environments

containing high chlorophyll and nitrogen compounds, having higher water temperature, and low dissolved oxygen and station depth. *Mycobacterium spp.* were not associated with environments having elevated wetland coverage or barren land (beaches). Full analysis of Coastal Bays data including *Vibrio vulnificus* will be conducted this Fall in preparation for publication.

Unresolved Issues:

- No funding for FY06
- 2nd technician needed to expand to full Chesapeake Bay
- Objective to initiate complimentary approach in South Carolina uncertain at the moment due to lack of funding

Budget Report:

50k was granted for this research in August of FY04 as a single, 2-year allotment for the period of FY04 and FY05. Funds were spent largely on supplies and equipment necessary for the project and to outfit new laboratory space ($40,500), outreach ($1,000), and travel ($2, 500). Additional laboratory space and large equipment expenses were covered by Oxford Laboratory base funds. No funding was received for FY06 with all expenses relating to this project covered by Oxford Laboratory base funds.

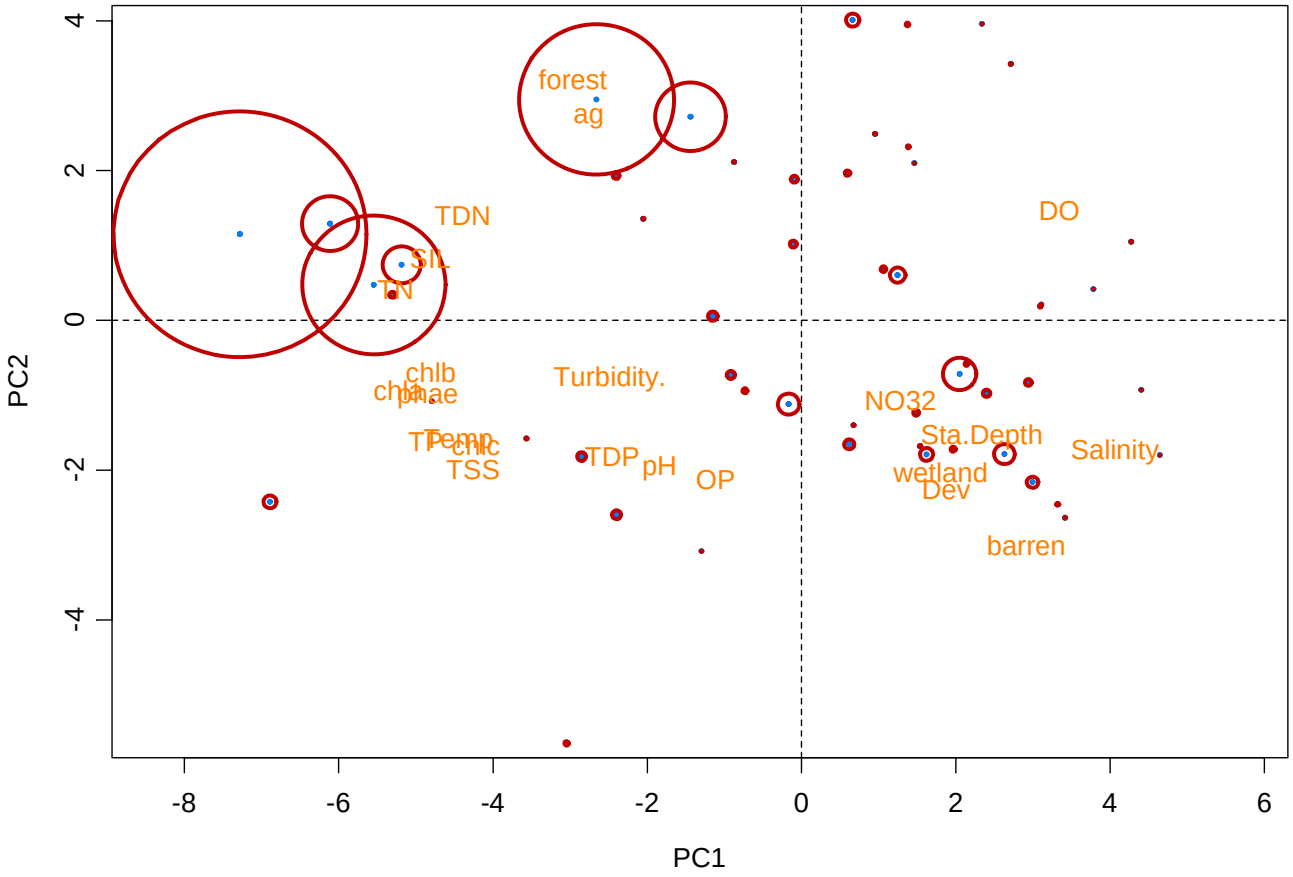

Figure 1 – Preliminary principal component analysis of *Mycobacterium spp.* density in relation to land use and water quality variables. O = individual data point with size of circle related to concentration of bacteria. PC1 explains the majority of the variance. Elevated concentrations of *Mycobacterium spp.* were positively associated with watersheds high in forest/ag lands and nitrogen loading, while furthest removed from deeper saline waters with elevated oxygen concentration.